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A reassessment of the inhibitory capacity of human FKBP38 on calcineurin

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Abstract The microbial peptidomacrolide FK506 affects many eukaryotic developmental and cell signaling programs via calcineurin inhibition. Prior formation of a complex between FK506 and intracellular FK506-binding proteins (FKBPs) is the precondition for the interaction with calcineurin. A puzzling difference has emerged between the mammalian multidomain protein *h*FKBP38 and other FKBPs. It was shown that *h*FKBP38 not only binds to calcineurin but also inhibits the protein phosphatase activity of calcineurin on its own [Shirane, M. and Nakayama, K.I. (2003) Nature Cell Biol. 5, 28–37]. Inherent calcineurin inhibition by *h*FKBP38 would completely eliminate the need for FK506 in controlling many signal transduction pathways. To address this issue, we have characterized the functional and physical interactions between calcineurin and *h*FKBP38. A recombinant *h*FKBP38 variant and endogenous *h*FKBP38 were tested both in vitro and in vivo. The proteins neither directly inhibited calcineurin activity nor affected NFAT reporter gene activity in SH-SY5Y and Jurkat cells. In addition, a direct physical interaction between calcineurin and *h*FKBP38 was not detected in co-immunoprecipitation experiments. However, *h*FKBP38 indirectly affected the subcellular distribution of calcineurin by interaction with typical calcineurin ligands, as exemplified by the anti-apoptotic protein Bcl-2. Our data suggest that *h*FKBP38 cannot substitute for the FKBP/FK506 complex in signaling pathways controlled by the protein phosphatase activity of calcineurin.

Keywords: Serine/threonine protein phosphatase; Calcineurin; Calmodulin; FK506-binding protein

1. Introduction

Calcineurin (Protein phosphatase 2B, CaN) links a reversible intracellular Ca^{2+}/calmodulin signal to the dephosphorylation reaction of many important proteins such as the transcription factors NFAT, MEF2, Elk-1, as well as NO synthase and the regulatory RII subunit of cAMP dependent protein kinase. Consequently, this enzyme couples calcium signaling to apoptosis and transcriptional control in the immune, nervous and cardiovascular systems [1–3]. When distributed into the cytoplasm of mammalian cells the microbial compounds FK506 and cyclosporin A (CsA) potently inhibit CaN by generating ternary complexes with their cellular targets FK506-binding proteins (FKBPs) and cyclophilins, respectively [4,5]. In immunosuppression, the protein component of the inhibitory complex of FK506 is thought to be FKBP12, thus, forming the FK506/FKBP12/CaN complex. The ubiquitously distributed FKBP12 is the prototypic FKBP-type peptidyl–prolyl *cis*/*trans* isomerase (PPIase) [6]. The FK506/FKBP12 complex, but not the drug alone binds to and tightly inhibits CaN in that the interface between the catalytic (CaNA) and the regulatory (CaNB) subunit of CaN is targeted by a composite drug–protein binding surface. In the case of FK506/FKBP12 and CsA/cyclophilin18, the contacting atoms do not directly interfere with the active site of CaNA. Rather, the restricted access of protein substrates to the active site underlying ternary complex formation constitutes the molecular basis for CaN inhibition [7–9].

Since these microbial drugs are not normal constituents of mammalian cells they are not available for the mammalian immunophilins to bind, and to perform a control of CaN activity from endogenously produced resources. This raises the question as to whether mammalian cells also contain compounds potentially targeting CaN in a FK506-like manner. Interestingly, there are derivatives of the microbial drugs that can inhibit CaN by their own, thus, circumventing the need for prior binding of the presenter proteins. It is a conformational change in the drug derivative that gives rise to a complete CaN interacting surface in the drug alone [10,11]. The conformational changes must allow replacement of important immunophilin–CaN contacting sites in the ternary complex with new drug-based interaction sites. A similar situation could be expected for the immunophilin component of the ternary complex. It is interesting to speculate, based on the small "effector domain" of FK506 [7], about the possibility of a peptide segment of a larger congener of FKBP12 capable of mimicking the FK506 "effector domain" in its CaN interfering conformation. Such a FKBP molecule would be expected to realize an inherent inhibitor of CaN.

Recently, inhibition of the RII phosphopeptide dephosphorylating activity of calcineurin was observed for an anti-hemagglutinin epitope immunoprecipitate of recombinant hemagglutinin epitope-tagged *h*FKBP38 giving rise to the theory that *h*FKBP38 by its own is able to mimic the composite surface of the FK506–FKBP12 complex. It was concluded that *h*FKBP38 forms the endogenous molecule of human cells that

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substitutes for the microbial ligand as represented by FK506 and CsA in CaN inhibition. However, adequate confirmation of CaN inhibition using untagged *h*FKBP38 in isolation is still lacking [12,13]. *h*FKBP38, a protein of 355 residues, belongs to those group of FKBP containing a PPIase domain, tetratricopeptide repeats and a putative Ca^{2+}/calmodulin binding site [6,14]. For a constitutive FKBP-like CaN inhibitor an increase of the threshold for entry to apoptosis was suggested [15]. It would be crucial to know whether *h*FKBP38 controls protein dephosphorylation by CaN, since the FKBP-like immunophilins were found to be involved in neuroprotection and neuroregeneration, processes that are tightly linked to apoptotic signals [16,17]. In this study, we test the finding that *h*FKBP38 is a CaN inhibitor by its own in vitro and in vivo. However, the present study shows that *h*FKBP38 neither interacts in vitro nor in vivo with CaN. Therefore *h*FKBP38 does not act as inherent inhibitor of CaN.

Furthermore, *h*FKBP38 inhibits CaN protein phosphatase activity in the presence of FK506. This observation supports the notion that *h*FKBP38 behaves overall in a manner similar to other immunophilins.

2. Materials and methods

Enzymes used: human $FKBP38^{1-336}$, human calmodulin, human Bcl-2, were recombinantly expressed by using a pET28a-vector in *E. coli* Rosetta™ cells. Human calcineurin was recombinantly expressed and purified according Mondragon et al. [18]. Maltose-binding protein–Bcl-2 fusion protein was purchased from Sigma (Deisenhofen, Germany). The *h*FKBP38 antibody was an affinity purified section 4 polyclonal rabbit antibody against the purified *h*FKBP38 domain (amino acids 1–166). Peptide substrates used were obtained from Bachem (Heidelberg, Germany). FK506 was purchased from calbiochem (La Jolla, CA).

2.1. Calcineurin activity assay

The calcineurin phosphatase assay was performed as described by Baumgrass et al. [19]. Direct inhibition of 5 nM recombinant human calcineurin by $hFKBP38^{1-336}$ was measured in the presence of 50 nM and 1 μM CaM at various concentrations of the PPIase (0–20 μM). Inhibition of 5 nM calcineurin by $hFKBP38^{1-336}$ was assayed at constant concentrations of 30 μM FK506 and 20 μM CaM in dependence on the $hFKBP38^{1-336}$ concentration. Due to the tight interaction between $hFKBP38^{1-336}$ and FK506, the concentration of the complex is almost identical to the $hFKBP38^{1-336}$ concentration used. Calcineurin activity was expressed relative to a reference without effectors. Error bars represent S.D. from the mean ($n = 3$).

2.2. Measurement of PPIase activity

PPIase activity was determined using protease-coupled assays as described elsewhere [20]. Typically, $hFKBP38^{1-336}$ PPIase activity was measured in a reaction mixture containing 1 μM $hFKBP38^{1-336}$, 5 μM recombinant human CaM and 5 mM $CaCl_2$. $hFKBP38^{1-336}$ was tested using the oligopeptide succinyl-AFPF-4-nitroanilide as substrate.

2.3. Protein–protein interaction in vitro

Native gel electrophoresis experiments; Native PAGE was performed according to Laemmli's gel system without SDS and β-mercaptoethanol at a constant temperature of 4 °C. CaM-binding assay; CaM–Sepharose (Amersham-Pharmacia Biotech) was preequilibrated in Ca^{2+}-containing buffer A (25 mM Tris/HCl, pH 7.5, 200 mM NaCl, 1 mM DTT, 2 mM $CaCl_2$). Subsequently, 1 ml crude *E. coli* lysate containing overexpressed $hFKBP38^{1-336}$ was incubated with CaM–Sepharose. After washing, bound proteins were eluted with buffer A containing 2 mM EGTA. Samples were subjected to 12.5% SDS–PAGE and analysed by Coomassie Blue staining. Co-immunoprecipitation; Cell lysis and co-immunoprecipitation experiments were performed according to manufactures protocols of the immunoprecipitation starter kit (Amersham Bioscience). Bcl-2 binding assay; 40 μl of 6 μM maltose-binding protein–Bcl-2 fusion protein (MBP–Bcl-2) was

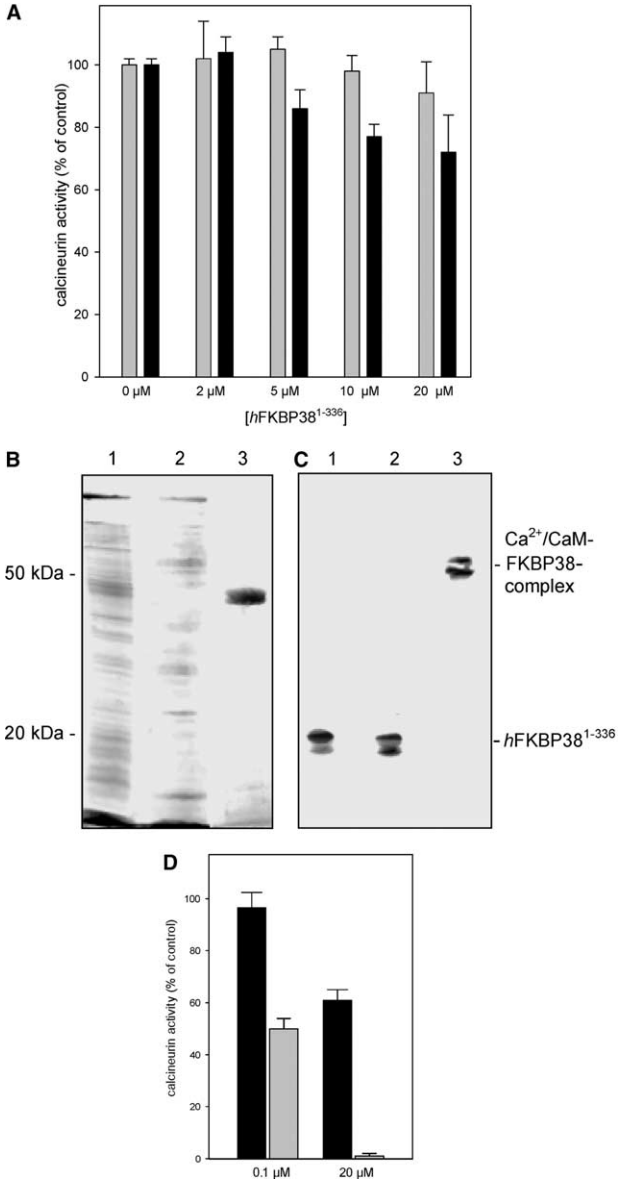


Fig. 1. Effect of *h*FKBP38 on CaN phosphatase activity. (A) CaN (5 nM) activity was measured in the presence of various concentrations of $hFKBP38^{1-336}$ in buffer containing either 50 nM CaM (black bars) or 1 μM CaM (grey bars) using [^{33}P]-labeled 19-residue phosphopeptide substrate (RII phosphopeptide). (B) *E. coli* cell lysate containing recombinant $hFKBP38^{1-336}$ was subjected to CaM–Sepharose in the presence of 2 mM $CaCl_2$. Bound protein was eluted by 2 mM EGTA and subsequently analysed by SDS–PAGE with Coomassie Blue staining; 1 – crude extract, 2 – washing step, 3 – eluat. (C) Native gel electrophoresis experiment was performed with samples containing 1 – $hFKBP38^{1-336}$, 2 – $hFKBP38^{1-336}$ preincubated with CaM, and 3 – $hFKBP38^{1-336}$ preincubated with CaM and $CaCl_2$. Samples were subjected to a 12.5% native gel. Migration of $hFKBP38^{1-336}$ was analysed by Western blot using rabbit anti-*h*FKBP38 antibody. (D) Residual CaN (5 nM) activity was determined in the presence of 30 μM FK506 and 20 μM CaM at varying concentrations of FKBP12 (grey bars) and *h*FKBP38 (black bars).

subjected to 40 μl amylose-resin (New England Biolabs, Beverly, MA) and incubated for 30 min. Thereafter, beads were washed twice with buffer B (25 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM DTT) and incubate with CaN. Subsequently, beads were incubated for 1 h with 40 μl reaction mixture containing Ca^{2+}/CaM, *h*FKBP38$^{1-336}$ and Ca^{2+}/CaM, *h*FKBP38$^{1-336}$, Ca^{2+}/CaM and GPI1046, FKBP38$^{1-336}$ or FKBP12. Bcl-2-bound CaN was eluted with 300 mM maltose, subjected to 12.5% SDS–PAGE and analysed by Western blotting using mouse anti-CaN antibody.

2.4. NFAT reporter gene assay

To test the effect of *h*FKBP38$^{1-336}$ overexpression on CaN phosphatase activity SH-SY5Y and Jurkat cells transfected with NFAT-luciferase reporter plasmid (Stratagene, Netherlands) were co-transfected by electroporation (Amaxa, Cologne, Germany) either with pcDNA4/HisMax C vector or a construct of this vector containing the sequence of *h*FKBP38. SH-SY5Y cells were cultured in DMEM (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine and 10% (v/v) heat-inactivated FCS in a humidified incubator at 37 °C in 10% (v/v) CO_2. Jurkat cells were cultured in RPMI 1640 with 10% FCS and 2 mM L-glutamine at 37 °C in 5% CO_2. Cells were stimulated with 2 μM ionomycin for 5 h. After cell lysis the protein content of each lysate was determined by the Bradford method. Equivalent amounts of protein were applied to determine the level of the extracted luciferase from the cells by bioluminescence measurement using the luciferase assay system (Promega, Mannheim, Germany). In addition, a β-galactosidase plasmid was co-transfected as internal standard.

2.5. Immunohistochemistry

SH-SY5Y cells grown on coverslips were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature. After washing in PBS cells were permeabilized 5 min on ice with 0.2% Triton X-100 in PBS containing 10% FCS, blocked with PBS containing 10% FCS for 1 h, incubated with PBS containing primary antibody (1:50 dilution for hamster anti-human Bcl-2 Ab (BD Biosciences Pharmingen)), 1:50 dilution for mouse anti-human CaN Ab (Sigma) and incubated with PBS containing secondary antibody (1:100 dilution for FITC-conjugated goat anti-mouse antibody IgG and 1:100 dilution for Cy5-conjugated goat anti-hamster antibody (Biomol, Hamburg, Germany)) and 10% FCS for 30 min. Cells were analysed by confocal laser scanning microscopy (Carl Zeiss LSM 410) at 750-fold magnification.

3. Results and discussion

3.1. Effect of hFKBP38 on CaN phosphatase activity in vitro

Human FKBP38 was previously identified for being an intrinsic CaN inhibitor by measurement of CaN activity after applying immunoprecipitated HA-tagged *h*FKBP38 expressed in HeLa cells [12]. Unfortunately, this report lacked a determination of inhibition constants, *h*FKBP38 concentration applied in the phosphatase assay or other biochemical data verifying the described interaction.

Therefore, we tested with purified proteins the inhibition of CaN activity by *h*FKBP38$^{1-336}$ in the absence of FK506 in order to determine the inhibition constant of this interaction. Calcineurin activity was measured using a scintillation proximity assay according to Baumgrass et al. [19]. A [^{33}P]-labeled biotinylated 19-residue peptide of a partial sequence of the RII subunit of the bovine PKA (RII phosphopeptide) was used as substrate. When performed in the presence of 50 nM Ca^{2+}/CaM a slight decrease in CaN activity was found at high concentrations of *h*FKBP38$^{1-336}$ (Fig. 1A, black bars). This small effect on CaN activity was abolished at 1 μM CaM and was found to be strictly dependent on the CaM concentration (Fig. 1A, grey bars). These findings are in strict contrast to previously reported data [12].

Recent data from our lab indicate that *h*FKBP38 interacts with CaM in a calcium-dependent manner (Fig. 1B and C). Thus, both Ca^{2+}/CaM-binding proteins, CaN and *h*FKBP38, may compete for the same cofactor in the CaN activity assay resulting in a weak CaN inhibition but only under Ca^{2+}/CaM limiting conditions. To gain insight in the putative presenter protein function of *h*FKBP38, CaN activity was investigated in the presence of the FK506 and *h*FKBP38$^{1-336}$. At 20 μM complex concentration residual CaN activity was about 60% of the control (Fig. 1D). Therefore, the potency of the gain-of-function, which is reported to be critical for the immunosuppressive activity of FK506, is similar to that observed for the FK506/FKBP52 complex, and about 200-fold lower than that for the FK506/FKBP12 complex [14].

In addition, we tested whether CaN influences the PPIase activity of *h*FKBP38 in a standard assay [20] using 1 μM *h*FKBP38$^{1-336}$, 5 μM CaM, 5 mM Ca^{2+} and CaN concentrations in a range of 200 nM–2 μM. We were not able to detect any influence of CaN on *h*FKBP38 activity (data not shown).

3.2. Effect of hFKBP38 on CaN phosphatase activity in vivo

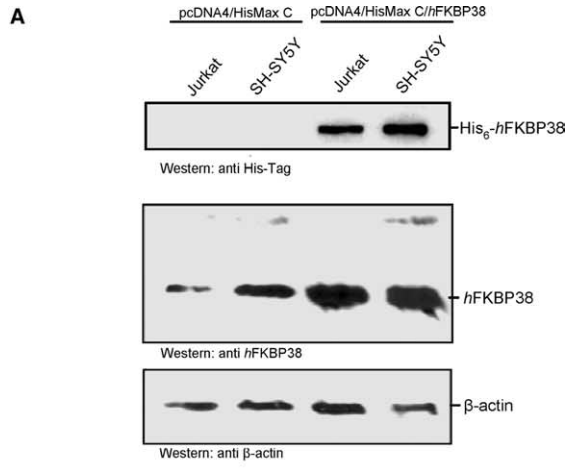
Based on these findings, we were interested in the effect of *h*FKBP38 on CaN activity in vivo. Therefore, we established a NFAT reporter gene assay in Jurkat and SH-SY5Y cells examining the CaN activity in living cells. Cells were co-transfected either with pcDNA4/HisMax C vector or a construct of this vector containing the full length sequence of *h*FKBP38 (Fig. 2A). In order to raise intracellular calcium levels and activate CaN activity cells were stimulated with 2 μM ionomycin for 5 h. As negative control stimulated cells were treated with the specific CaN inhibitor CsA. GPI1046 was used to affect *h*FKBP38. Due to application of the calcium ionophore ionomycin NFAT dephosphorylation increases. In the presence of CsA dephosphorylation levels were comparable to those in unstimulated cells. In SH-SY5Y cells neither overexpression of *h*FKBP38 nor addition of the immunophilin ligand GPI1046 had a significant effect on CaN phosphatase activity. However, in Jurkat cells CaN activity increases upon *h*FKBP38 overexpression, whereas application of GPI1046 resulted in a slight decrease of NFAT dephosphorylation (Fig. 2B and C). The difference between both cell lines seems to be a result of different levels of endogenous *h*FKBP38 (Fig. 2A). Assuming particular high concentrations of *h*FKBP38 in neuronal cells as previously reported [14], *h*FKBP38 overexpression would cause small additional effects. These results imply that *h*FKBP38 does not inhibit CaN protein phosphatase activity in vivo.

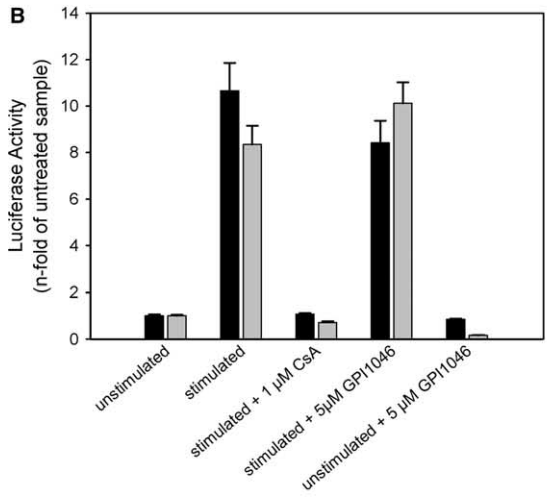
3.3. Physical hFKBP38/CaN interaction

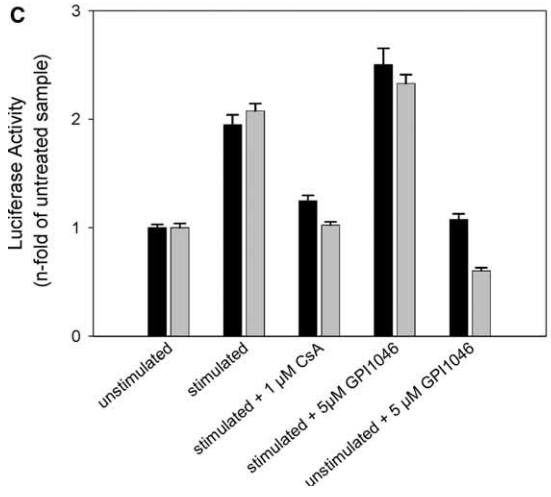
In attempt to investigate a physical interaction between endogenous CaN and *h*FKBP38 we performed co-immunoprecipitation experiments. As shown in Fig. 3A and B, *h*FKBP38 did not co-precipitate with CaN neither in the presence nor the absence of calcium. In contrast, CaM did co-precipitate with CaN in the presence of calcium. To summarize results obtained from the enzyme activity assays and the co-immunoprecipitation experiments, *h*FKBP38 and CaN interact both with CaM and therefore may compete for this protein under CaM limiting conditions in the cell, but do not interact physically with each other.

3.4. Influence of hFKBP38 on CaN–Bcl-2 interactions

To verify results obtained with NFAT reporter gene assay in Jurkat cells that are not based on direct interaction between *h*FKBP38 and CaN, we were interested in determining whether Bcl-2 might serve as the missing link between *h*FKBP38 and the regulation of CaN activity, because reported data [12,21] describe interactions of CaN and *h*FKBP38 with Bcl-2.







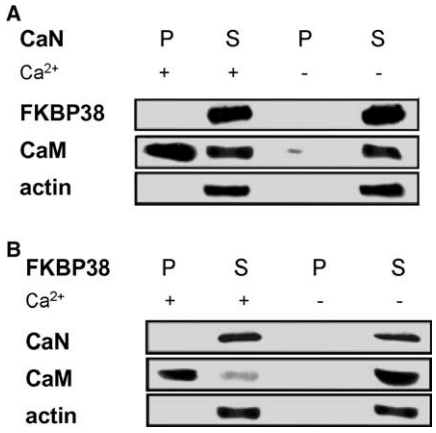


Fig. 3. CaN does not interact with *h*FKBP38. Co-immunoprecipitation; SH-SY5Y cell lysate was incubated with (A) mouse anti-calcineurin antibody and (B) rabbit anti-*h*FKBP38 antibody. Antibody/protein complexes were bound to protein G sepharose. Samples were washed. Pellet (P) and Supernatant (S) were subjected to SDS–PAGE and analysed by Western blot using mouse anti-CaM antibody and (A) rabbit anti-*h*FKBP38 antibody or (B) mouse anti-calcineurin antibody. β-Actin was used as loading control.

Using a binding assay, where a MBP–Bcl-2 fusion protein was bound to amylose resin, direct binding of CaN to Bcl-2 was observed. *h*FKBP38 did not affect this interaction. However, *h*FKBP38$^{1\text{-}336}$ was able to interfere with CaN–Bcl-2 interactions upon addition of Ca^{2+}/CaM. The effect of *h*FKBP38 application on CaN–Bcl-2 interaction was abolished in the presence of the neuroimmunophilin ligand GPI1046. Neither *h*FKBP12 nor CaM alone did affect this interaction (Fig. 4A).

In order to verify whether the observed disruption of the Bcl-2–CaN interaction by *h*FKBP38 has an effect on CaN subcellular distribution, we performed an experiment observing the localization of Bcl-2 and CaN in SH-SY5Y cells (Fig. 4B). In order to increase cellular calcium concentrations cells were stimulated by ionomycin. The neuroimmunophilin ligand GPI1046 was applied to stimulated cells to affect *h*FKBP38 and therefore preventing disruption of Bcl-2/CaN interactions. In unstimulated cells Bcl-2 and CaN are predominantly co-localized. Upon calcium rise induced by calcium ionophore ionomycin CaN mainly redistributed from Bcl-2. Application of GPI1046 prevents CaN redistribution due to intracellular Ca^{2+} rise, indicating that *h*FKBP38 may cause an increased proportion of CaN in the cytosol and therefore led to higher

Fig. 2. *h*FKBP38 does not affect NFAT reporter gene activity. (A) SH-SY5Y and Jurkat cells were transfected with NFAT-luciferase reporter plasmid and co-transfected either with pcDNA4/HisMax C vector (grey bar) or a construct of this vector containing the sequence of *h*FKBP38 (black bar). Expression of *h*FKBP38 was analysed by Western blot using mouse anti-HisTag antibody. Endogenous and artificially expressed hFKBP38 was analysed by Western blot using rabbit anti-*h*FKBP38 antibody. β-Actin was used as loading control. Jurkat (B) and SH-SY5Y cells (C) were stimulated with 2 μM ionomycin for 5 h. In some cases, cells were preincubated with the neuroimmunophilin ligand GPI1046 and the calcineurin inhibitor CsA for 30 min. After cell lysis, the amount of the extracted luciferase from the cells was determined by bioluminescence measurement. Activities are expressed as *n*-fold over basal. The data presented are means ± S.D. of three independent experiments.

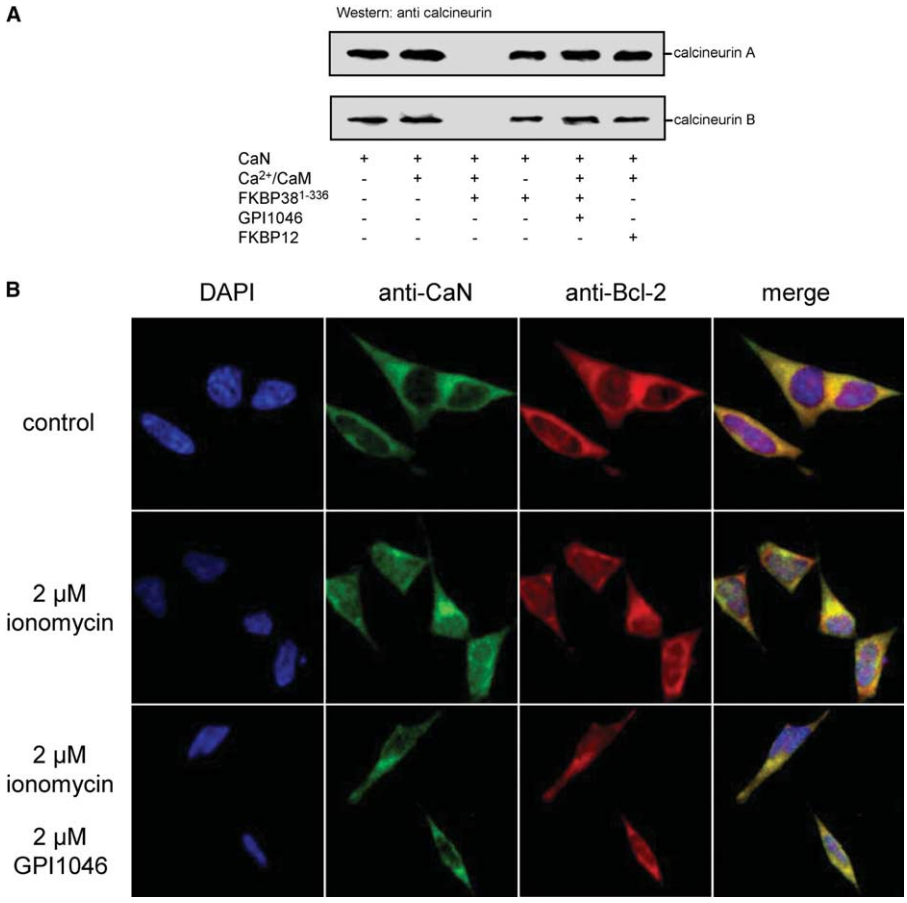


Fig. 4. Ca^{2+}/CaM complexed *h*FKBP38$^{1-336}$ disrupts Bcl-2–CaN interaction. (A) MBP–Bcl-2 fusion protein was immobilized on amylose beads and incubated with CaN. *h*FKBP38$^{1-336}$ did not interfere with the detected CaN/Bcl-2 interaction. In the presence of Ca^{2+}/CaM *h*FKBP38$^{1-336}$ disrupted CaN/Bcl-2 complexes. Upon addition of the neuroimmunophilin ligand GPI1046 this effect was abolished. *h*FKBP12 did not interfere with the CaN/Bcl-2 interaction. Bcl-2-bound CaN was eluted with 300 mM maltose, subjected to 12.5% SDS–PAGE and analysed by Western blotting using mouse anti-CaN antibody. (B) Subcellular distribution of CaN and Bcl-2 in SH-SY5Y neuroblastoma cells was analysed by immunostaining with FITC-conjugated goat anti-mouse antibody against mouse anti-CaN antibody and Cy5-conjugated goat anti-hamster antibody against hamster anti-Bcl-2 antibody. Nuclei were stained with DAPI. Cells were treated for 5 h with 2 µM ionomycin and 2 µM GPI1046. Bcl-2 and CaN co-localize in unstimulated SH-SY5Y cells. After application of the calcium ionophore ionomycin no co-localization is observed, whereas after addition of 2 µM GPI1046 CaN co-localizes with Bcl-2.

CaN activity detected in the presence of high cellular calcium concentrations.

4. Conclusion

The results obtained with co-immunoprecipitation experiments, CaN activity assay, and the NFAT reporter gene assay show that *h*FKBP38 cannot function as a binding partner or inherent inhibitor of CaN. Upon formation of the immunophilin/immunosuppressant complex CaN protein phosphatase activity is inhibited. Therefore, *h*FKBP38 behaves as other immunophilins by inhibiting CaN in complex with the immunosuppressive drug whereas the protein by its own does not.

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